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Peptide Research

Cyclic Peptide Template Combinatorial Libraries: Synthesis and Identification of Chymotrypsin Inhibitors

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ABSTRACT

A cyclic peptide template combinatorial library in a positional scanning format, composed of three positional libraries, was synthesized using solid-phase chemistry and four orthogonal protecting groups (Fmoc, Boc, Dde, OAll). The cyclic peptide template is composed of three lysine residues and one glutamic acid residue. The chemical diversity was introduced by acylating the ε-amino groups of the lysine residues using 10 carboxylic acids in addition to the 20 proteinogenic amino acids. The library components have been shown to be stable towards proteolytic degradation. Compounds with chymotrypsin inhibitory activity were identified through the screening of this library.

INTRODUCTION

Synthetic combinatorial library approaches are increasingly appreciated as useful tools for basic research and drug discovery programs. As first developed for peptides (18,23,29), the general concept of combinatorial libraries for the identification of novel ligands involves the generation of all possible sequence combinations for a peptide of a given length (e.g., 64 000 000 for hexapeptides composed only of the 20 proteinogenic amino acids), in connection with a screening process that enables the identification of unique, highly active peptides in the presence of millions of less active or inactive peptides. As the individual synthesis and testing of such large numbers of peptides is currently impossible, methods of synthesizing mixtures of millions of peptides have recently been shown to be not only feasible, but also broadly useful, since they have been successfully used to identify a variety of peptide ligands (e.g., antigenic determinants, receptor binding peptides, enzyme substrates and inhibitors) (16,21,24, reviews). A number of approaches for the generation of peptide libraries have been presented. Using recombinant DNA techniques, large numbers of short peptides can be expressed randomly in a fusion phage vector system (46). This method, however, remains restricted to the 20 proteinogenic amino acids as building blocks for the peptides. Chemical approaches to the synthesis of peptide libraries (18,23, 29), on the other hand, allow for the incorporation of nonproteinogenic (1) and D-amino acids (9,28,39), as well as chemically modified amino acids (45).

Chemically synthesized peptide libraries can be immobilized on a solid support, such as plastic pins (18), resin beads (29) or cotton (11,12), or are composed of peptide mixtures in solution (23). Whereas the screening of immobilized libraries is limited to solid-phase binding assays, soluble synthetic combinatorial libraries (SCLs) can be screened in virtually all relevant bioassays. The first SCL presented (23) was composed of 400 separate hexapeptide mixtures, represented as O_1O_2XXXX, in which the first two positions (O) were individually defined with one of the 20 proteinogenic amino acids, and the remaining four positions (X) were occupied by approximately equimolar mixtures of amino acids. In order to identify highly active components of the library, the most active of the 400 peptide mixtures in a given bioassay were carried through an iterative synthesis and screening process, during which all positions of the active sequences were successively defined.

An alternative SCL approach, termed positional scanning SCL (8,38,39), enables the identification of active compounds in a single screening. A hexapeptide positional scanning SCL is made up of six independent positional libraries, represented as O_1XXXXX, XO_2XXXX, XXO_3XXX, $XXXO_4XX$, $XXXXO_5X$ and $XXXXXO_6$. Each of the positional libraries represents the same set of peptides, with the difference between them being the location of the defined position (O). When used in concert, the screening of the entire positional scanning SCL provides information about the most effective amino acids in each position for a given ligand-receptor interaction. Synthesis of all possible combinations of the most effective amino acids in each position results in individual peptides, which are then tested in order to verify the library screening data, as well as to establish the activity of individual peptides.

The generally poor oral availability and rapid enzymatic breakdown of linear L-amino acid peptides make them inferior drug candidates relative to other

organic (i.e., non-peptide) compounds. Therefore, the major research focus in the field of combinatorial chemistry is currently on the development of chemically modified (37), as well as peptidomimetic and non-peptide, libraries (19).

The reduced flexibility of cyclic peptides is thought to increase their potential for high affinity binding to various acceptor molecules. Furthermore, cyclic peptides have been reported to be resistant to enzymatic degradation (7,44). Cyclic peptides have also been used as templates for the construction of conformationally defined molecules with the aim to generate protein-like structures, as well as "surface mimetics" of discontinuous binding sites of proteins (48). Various methods of peptide cyclization on the solid support through lactam formation have been presented (25,30,32,40–42,47), which have also been used for the synthesis of cyclic peptide mixtures (5). These methods provide the methodological tools for the synthesis of cyclic peptide combinatorial libraries. Sequence dependence in the yield of cyclic monomer (35), however, may result in disproportional representation of individual peptides within the cyclic peptide library.

Based on the above premises, a combinatorial library based on a cyclic peptide template (Figure 1) was designed and synthesized. The chemical diversity represented by this library was extended by utilizing carboxylic acids as building blocks (31) in addition to the proteinogenic amino acids. The components of this library are expected to be less prone to enzymatic degradation than their linear analogs.

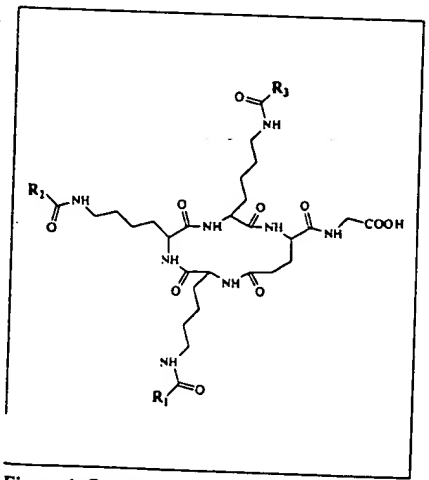

Figure 1. Structure of the cyclic template. "R" represents amino acid or carboxylic acid residues.

MATERIALS AND METHODS

General

Boc-Gly-PAM (Boc = *t*-butyloxycarbonyl; PAM = phenylacetamidomethyl) resin and Fmoc-protected amino acid derivatives (Fmoc = 9-fluorenylmethoxycarbonyl) were obtained from Bachem California (Torrance, CA, USA), except Fmoc-Lys(Dde) and Dde-Lys(Fmoc) [Dde = (1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl], which were purchased from Calbiochem/Novabiochem USA (La Jolla, CA, USA), and Fmoc-Glu(OAll) [OAll = allyl ester], which was from SNPE (Princeton, NJ, USA). *N*-Hydroxybenzotriazole (HOBt), spectroscopic grade dimethylformamide (DMF), carboxylic acids, piperidine, acetic anhydride, pyridine, diisopropylethylamine (DIEA), hydrazine, diethyldithiocarbamic acid sodium salt (DEDTA), *N*-methylmorpholine (NMM), dimethylsulfoxide (DMSO), and tetrakis (triphenylphosphine)palladium(0) were obtained from Aldrich (Milwaukee, WI, USA). Diisopropylcarbodiimide (DIC) and benzotriazole-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP) were purchased from Chem Impex (Wood Dale, IL, USA), trifluoroacetic acid (TFA) from Halocarbon (River Edge, NJ, USA), triisobutylsilane from Fluka Chemical (Ronkonkoma, NY, USA) and dichloromethane (DCM) from Fisher Scientific (Fair Lawn, NJ, USA). Tosyllysylchloromethylketone (TLCK)-treated chymotrypsin and *N*-succinyl-L-phenylalanine-*p*-nitroanilide were obtained from Sigma Chemical (St. Louis, MO, USA). Hydrogen fluoride (HF) was purchased from Air Products (San Marcos, CA, USA). All chemicals and solvents were used as received without further purification.

Matrix-assisted laser desorption ionization was used for the generation of mass spectra on a MALDI instrument from Kratos Analytical (Ramsey, NJ, USA). Reverse-phase high performance liquid chromatography (RP-HPLC) was carried out using a System Gold instrument from Beckman Instruments (Fullerton, CA, USA).

Library Synthesis

(i) Sequence assembly

The library was synthesized by simultaneous multiple solid phase-peptide synthesis (20). The solid support (Boc-Gly-PAM polystyrene resin, 0.53 meq/g) was contained in 90 polypropylene mesh bags (100 mg resin per bag). After Boc-deprotection (55% TFA/DCM, 30 min), the resin bags were washed (3× DCM, 3× 5% DIEA/DCM, 3× DCM). The 90 resin bags were then shaken in 240 mL of 0.1 M Fmoc-Glu(OAll)/DIC/HOBt in DMF for 2 h, and washed (3× DMF, 3× DCM). After removal of the Fmoc group (20% piperidine/DMF, 20 min, wash: 3× DMF, 3× DCM), 30 of the 90 bags were shaken in 80 mL of 0.1 M Fmoc-Lys(Dde)/DIC/HOBt in DMF for 2 h, and washed (3× DMF, 3× DCM). The remaining 60 bags were shaken in 160 mL of 0.1 M Fmoc-Lys(Boc)/DIC/HOBt in DMF for 2 h, and washed (3× DMF, 3× DCM). The Fmoc-deprotection and coupling of the two different lysine derivatives was repeated twice, wherein Fmoc-Lys(Dde) was coupled to a different set of 30 bags in each coupling.

(ii) Allyl ester cleavage

Tetrakis(triphenylphosphine)palladium(0) (6.3 g) was dissolved in 540 mL of a mixture of DMF/acetic acid/NMM (5:1:0.5 vol/vol/vol), which had been purged with argon for 15 min. The 90 resin bags were vigorously shaken in this solution under argon for 3 h, and washed (3× DMF, 3× DCM, 3× 5% DIEA/DCM, 3× DCM, 3× DMF, 3× 0.02 *N* DEDTA/DMF, 3× DMF, 3× DCM).

(iii) Cyclization

After removal of the N-terminal Fmoc group, the 90 resin bags were shaken overnight in 240 mL of 0.1 M PyBOP/HOBt and 0.2 M DIEA in DMF, and washed (3× DMF, 3× DCM).

(iv) Incorporation of mixture (X) positions

After removal of the Boc group from the lysine side chains (55% TFA/DCM, 30 min), the 90 resin bags were shaken overnight in 230 mL of an 0.05 M equimolar mixture of 19 of the 20 proteinogenic amino acids [cysteine excluded, N^{α}-protection: Fmoc, side-chain protection: *t*-butyl ether (tBu) for serine, threonine and tyrosine; *t*-butyl ester (OtBu) for aspartic acid and glutamic acid; trityl (Trt) for histidine, asparagine and glutamine; Boc for lysine; 2,2,5,7,8-pentamethylchroman-6-

sulfonyl (Pmc) for arginine] and the 10 carboxylic acids shown in Figure 2, along with 0.05 M PyBOP/HOBt and 0.1 M DIEA in DMF, and washed (3× DMF, 3× DCM). After removal of the Fmoc group, the resin bags were shaken in 270 mL of a mixture of acetic anhydride/pyridine/DMF 1:2:3 (vol/vol/vol) for 60 min, and washed (3× DMF, 3× DCM).

(v) Incorporation of the defined (O) positions

After removal of the Dde group from the lysine side chains (2% hydrazine/DMF, 5 + 10 min; wash: 3× DMF, 3× DCM), the 90 resin bags were divided into 30 groups of three bags (each of the three bags in each group had the Lys(Dde) residue in another position). The three bags of each group were then shaken in 8 mL of a 0.1 M solution of one of the 20 Fmoc-L-amino acid, or one of the 10 carboxylic acids shown in Figure 2, along with DIC and HOBt, for 2 h, and washed (30 DMF, 30 DCM). After removal of the Fmoc group from the 60 bags to which an Fmoc-amino acid was coupled, these 60 bags were shaken in 180 mL of a mixture of acetic anhydride/pyridine/DMF 1:2:3 (vol/vol/vol) for 60 min, and washed (3× DMF, 3× DCM).

(vi) Deprotection of side chains

The 90 resin bags were shaken in 540 mL of a mixture of TFA/ DCM/ water/triisobutylsilane 50:30:10:10 (vol/vol/vol/vol) for 3 h, and washed (3× DCM, 3× 5% DIEA/DCM, 3× DCM).

(vii) Cleavage from the resin

The peptide mixtures were cleaved from the resin by treatment with 10% anisole/HF as described earlier (22). After cleavage, the peptide mixtures were extracted from the resin with 50% acetonitrile/water under sonication, lyophilized, re-lyophilized from 50% acetonitrile/water, dissolved in 15% DMSO/water at a concentration of 5 mg/mL, and frozen.

Synthesis of Individual Compounds Derived from the Library

Individual compounds of the formula *cyclo*[Lys(O_1)-Lys(O_2)-Lys(O_3)-Glu]-Gly-OH were synthesized stepwise using Boc-Gly-PAM resin as the solid support. Amino acids and carboxylic acids were coupled by means of DIC/HOBt (5 eq, 0.1 M solution in DMF, 2 h). After Boc-deprotection, Fmoc-Glu(OAll) was coupled, followed by coupling of Dde-Lys(Fmoc), removal of the Fmoc group of the ε-amino group of lysine (20% piperidine/DMF, 20 min) and coupling of the Fmoc-amino acid (side-chain protection as noted above) or carboxylic acid representing O_3. After Fmoc-deprotection and acetylation (only if O_3 was an amino acid), the Dde group was removed from the α-amino group of lysine (2% hydrazine/DMF, 5 + 10 min). The coupling of Dde-Lys(Fmoc), followed by coupling of an amino or carboxylic acid, was repeated twice, thus incorporating the amino or carboxylic acids representing O_2 and O_1, respectively. After cleavage of the allyl ester (as described for the library synthesis) and

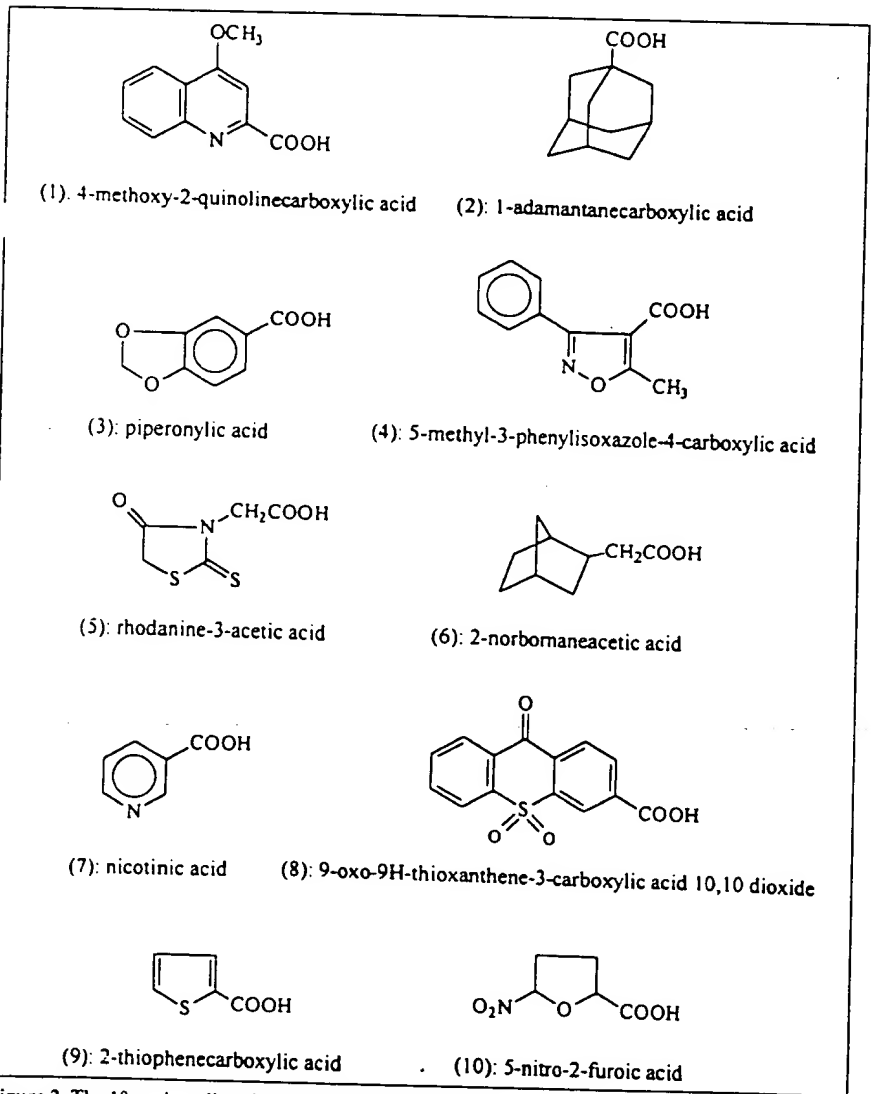

Figure 2. The 10 carboxylic acids used as building blocks for the library.

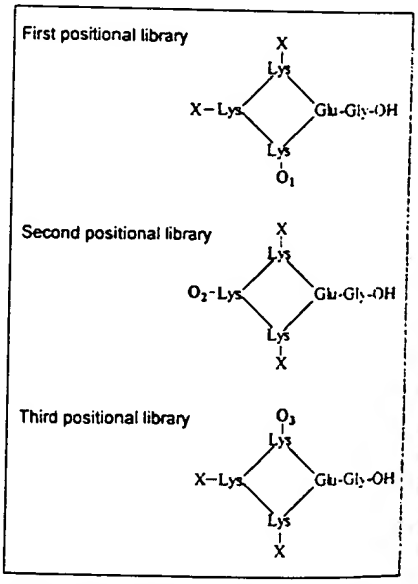

Figure 3. The positional scanning format of the library. X: mixture positions, O: individually defined positions.

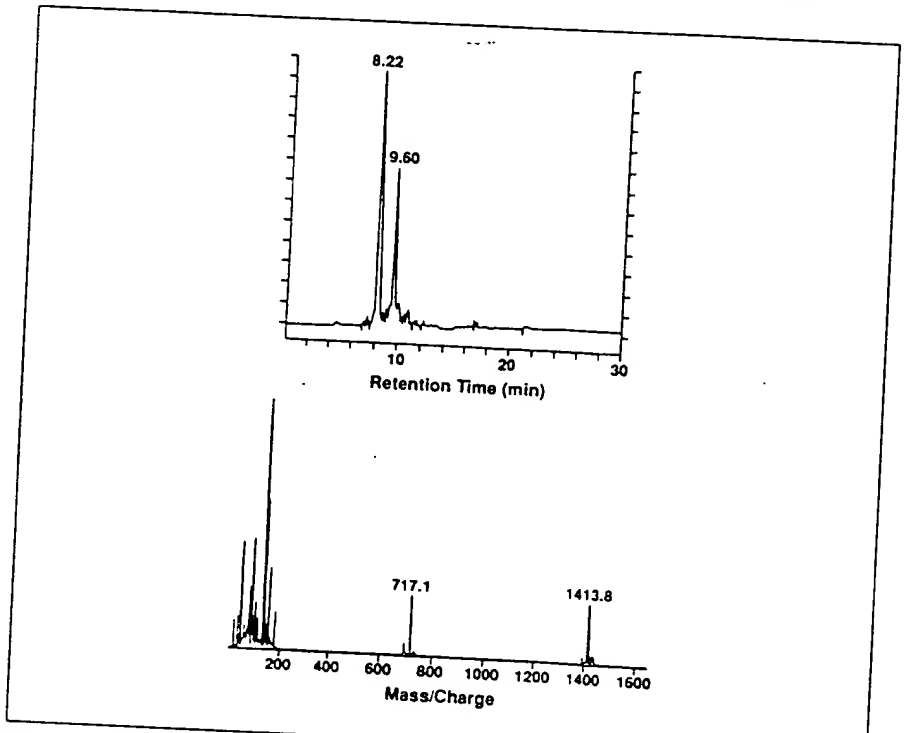

Figure 4. RP-HPLC profile (top) (conditions: Vydac C18; gradient: 5%–95% 0.1% TFA/acetonitrile in 0.1% TFA/water in 30 min; detection: 220 nm) and mass spectrum (bottom) of *cyclo*[Lys(Ac)-Lys(Ac)-Lys(Ac)-Glu]-Gly-OH.

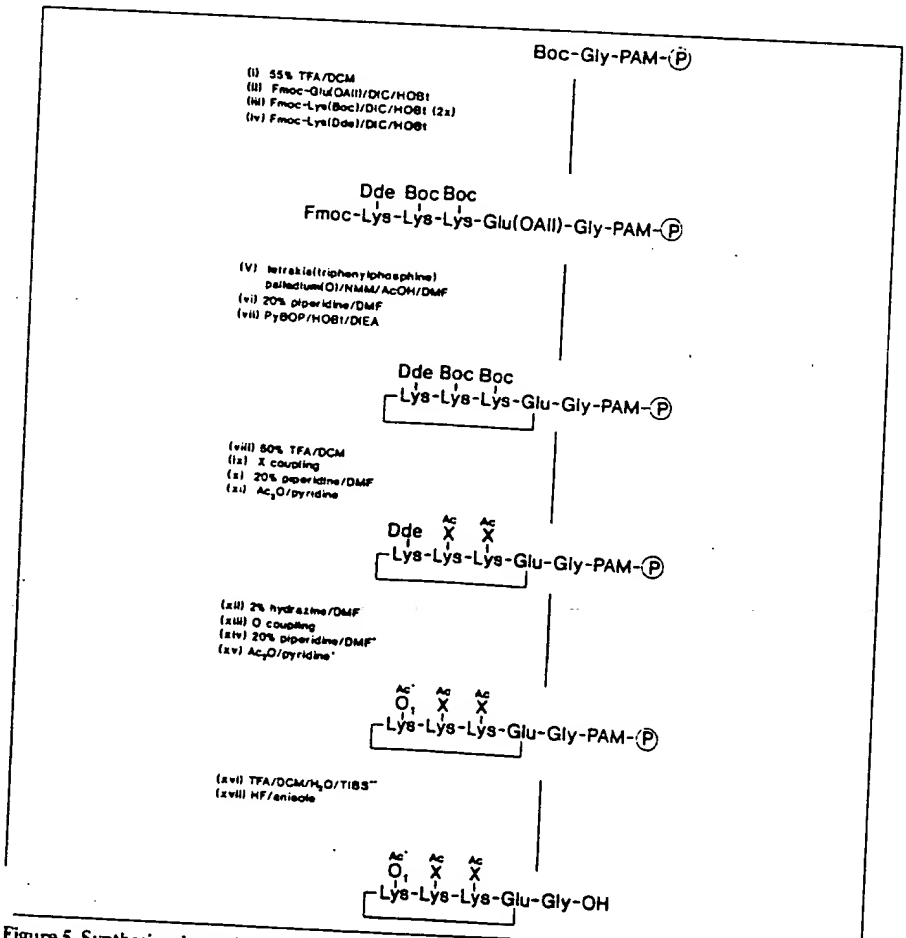

Figure 5. Synthesis scheme for the first positional library, *cyclo*[Lys(O_1)-Lys(X)-Lys(X)-Glu]-Gly-OH. *Only if O is an amino acid; **triisobutylsilane

removal of the N-terminal Dde group, the peptides were cyclized by means of PyBOP/HOBt (5 eq) and DIEA (10 eq) overnight. After deprotection of the side chains (TFA/ DCM/ water/triisobutylsilane 50:30:10:10, 3 h; only when trifunctional amino acids were in the O positions), the peptides were cleaved from the resin with HF, characterized by analytical RP-HPLC and mass spectroscopy, and purified by preparative RP-HPLC.

Chymotrypsin Inhibition Assay

The peptide mixture solutions (50 µL, 5 mg/mL) or solutions of individual peptides (50 µL, 1 mg/mL), 80 µL 0.1 M Tris-buffer containing 0.025 M $CaCl_2$ (pH 7.8) and 20 µL TLCK-treated chymotrypsin (from bovine pancreas) solution (0.1 mg/mL 0.0025 M HCl) were incubated in the wells of a 96-well assay plate at 37°C for 30 min. Then, 100 µL of the chromogenic substrate (*N*-succinyl-L-phenylalanine-*p*-nitroanilide, 2 mg/mL buffer) were added. After incubation at 37°C for 45 min, absorbencies (ODs) of the solutions were read at 405 nm using a Titertek Multiscan (Flow Laboratories, McLean, VA, USA) and expressed as % inhibition, which was calculated according to the following formula:

$$\% \text{ inhibition} = \{1 - [(OD_{sample} - OD_{blank})/(OD_{control} - OD_{blank})]\} \times 100$$

"Control" refers to a sample without peptide, and "blank" to a sample without chymotrypsin. Serial dilutions of peptide solutions were used to determine the IC_{50} values (peptide concentration necessary for 50% inhibition).

RESULTS AND DISCUSSION

Library Synthesis

The cyclic peptide template library has a positional scanning format (38) and is composed of three independent positional libraries (Figure 3), which are represented as *cyclo*[Lys(O_1)-Lys(X)-Lys(X)-Glu]-Gly-OH, *cyclo*[Lys(X)-Lys(O_2)-Lys(X)-Glu]-Gly-OH and *cyclo*[Lys(X)-Lys(X)-Lys(O_3)-Glu]-Gly-OH. X represents mixture positions occupied by an approximately equimolar mixture of 19 of the 20 proteinogenic amino acids (cysteine excluded), along with 10 additional carboxylic acids (Figure 2). These

carboxylic acids introduce structural elements that are not represented by the proteinogenic amino acids (e.g., heterocycles, xanthene, adamantane and norbornane structures). O represents individually defined positions occupied by one of the 19 amino acids, or one of the 10 carboxylic acids. Each positional library consists of 30 separate peptide mixtures, with each mixture composed of 841 (29^2) individual compounds.

An important prerequisite for the synthesis of this library was the availability of orthogonal (i.e., selectively cleavable) protecting groups for the α- and ε-amino groups of lysine, as well as the γ-carboxylic function of glutamic acid. The allyl ester, as well as the Boc and Fmoc groups, can be cleaved independently. The Dde group is stable in 20% piperidine, which is used for the deprotection of the Fmoc-protected α-amino groups of lysine, yet is readily cleaved in 2% hydrazine in DMF (4).

Before starting the library synthesis, the unsubstituted cyclic peptide template, *cyclo*[Lys(Ac)-Lys(Ac)-Lys(Ac)-Glu]-Gly-OH (molecular weight: 695), was synthesized and analyzed. The RP-HPLC profile and mass spectrum of the crude peptide are shown in Figure 4. The two major signals on the mass spectrum represent the sodium-adducts of the monomer (695 + 23 = 718) and dimer (695 × 2 + 23 = 1413) of the peptide (the signals below 200 are matrix-related). The two major HPLC-peaks with retention times of 8.22 min (42%) and 9.60 min (26%) were isolated and found to represent the monomeric cyclic peptide (8.22 min) and the dimer (9.6 min). These results indicate that the desired monomeric cyclic peptide template is clearly the major product of this synthesis.

The strategy for the synthesis of the first positional library, *cyclo*[Lys(O_1)-Lys(X)-Lys(X)-Glu]-Gly-OH, is outlined in Figure 5. Following assembly of the linear template (Fmoc-Lys(Dde)-Lys(Boc)-Lys(Boc)-Glu(OAll)-Gly-PAM-resin), the γ-allyl ester of glutamic acid was cleaved using the soluble catalyst tetrakis(triphenylphosphine)palladium(0). This was followed by Fmoc-deprotection of the N-terminal amino group. The template was cyclized through lactam formation between the N-terminal amino group and the γ-carboxylic function of glutamic acid. It should be noted that at this stage of the synthesis, all of the peptides were identical, thus avoiding the above-mentioned sequence-dependent bias associated with the cyclization of peptide mixtures. Once the cyclic template had been generated, the chemical diversity was introduced by acylating the ε-amino groups of the three lysine residues. First, the Boc groups were removed from the ε-amino groups of the lysine residues in positions two and three, and the mixture (X) positions were introduced by simultaneous acylation of both ε-amino groups using a total of 1.2 equivalents of an equimolar mixture of 19 of the 20 proteinogenic amino acids (cysteine excluded), along with the 10 additional carboxylic acids listed in Figure 2. By using only 1.2 equivalents of acylation mixture, over- or underrepresentation of some amino or carboxylic acids, due to differences in acylation rates, can be minimized (27). In order to determine the incorporation ratio of amino acids with different acylation rates (e.g., alanine, phenylalanine and isoleucine), the peptide mixture Lys(Ala/Phe/Ile)-Lys(Ala/Phe/Ile)-NH_2, composed of nine peptides, was synthesized using the method described above for the simultaneous acylation of both lysine side chains with 1.2 equivalents of an equimolar mixture of alanine, phenylalanine and isoleucine. The individual peptides making up this peptide mixture were separately synthesized as controls in order to identify the components within the mixtures according to their RP-HPLC retention times. The RP-HPLC profile of the mixture indicated

Table 1. Ratio of Individual Peptides in the Peptide Mixture Lys(Ala/Ile/Phe)-Lys(Ala-Ile/Phe)-NH_2[a]

Peptide/Peptide Pair	Peak Area Ratio
Lys(Ala)-Lys(Ala)-NH_2	1
Lys(Ile)-Lys(Ile)-NH_2	0.98
Lys(Ala)-Lys(Ile)-NH_2 / Lys(Ile)-Lys(Ala)-NH_2	1.84
Lys(Phe)-Lys(Phe)-NH_2	2.22
Lys(Ile)-Lys(Phe)-NH_2 / Lys(Phe)-Lys(Ile)-NH_2	2.96
Lys(Ala)-Lys(Phe)-NH_2 / Lys(Phe)-Lys(Ala)-NH_2	3.70

[a]Determined by RP-HPLC—Conditions: Vydac C_{18}; gradient: 5-65% 0.1%TFA/acetonitrile in 0.1% water/acetonitrile in 30 min; detection: 220 nm.

Table 2. Chymotrypsin Inhibitory Activity of Individual Compounds Identified Through the Library Screening

Compound	IC_{50} [μM]
cyclo[Lys(3)-Lys(3)-Lys(3)-Glu]-Gly-OH[a]	68
cyclo[Lys(3)-Lys(3)-Lys(9)-Glu]-Gly-OH	63
cyclo[Lys(3)-Lys(9)-Lys(3)-Glu]-Gly-OH	55
cyclo[Lys(3)-Lys(9)-Lys(9)-Glu]-Gly-OH	60
cyclo[Lys(9)-Lys(9)-Lys(9)-Glu]-Gly-OH	94
cyclo[Lys(9)-Lys(9)-Lys(3)-Glu]-Gly-OH	57
cyclo[Lys(9)-Lys(9)-Lys(3)-Glu]-Gly-OH	57
cyclo[Lys(9)-Lys(3)-Lys(9)-Glu]-Gly-OH	51
cyclo[Lys(9)-Lys(3)-Lys(3)-Glu]-Gly-OH	52
Ac-Lys(9)-Lys(3)-Lys(9)-Gly-OH	432
Ac-Lys(9)-Lys(3)-Lys(3)-Gly-OH	496
Ac-Lys(3)-Lys(9)-Gly-OH	692
Ac-Lys(3)-Lys(3)-Gly-OH	736
Ac-Lys(9)-Gly-OH	1060
Ac-Lys(3)-Gly-OH	1619
Ac-ygyyyr-NH_2[b]	36
Chymostatin	1.64

[a]3: piperonylic acid; 9: 2-thiophenecarboxylic acid (see Figure 2)
[b]Lower-case letters indicate D-amino acids

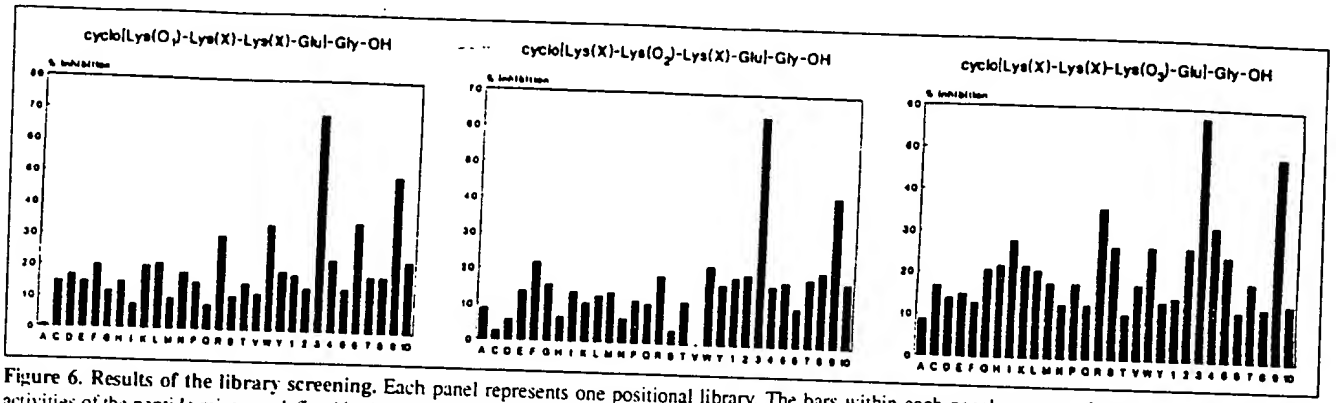

Figure 6. Results of the library screening. Each panel represents one positional library. The bars within each panel represent the chymotrypsin inhibitory activities of the peptide mixtures defined in the (O) position with the amino acid or carboxylic acid specified on the x-axis (A through Y and 1 through 10).

a balanced representation of all nine components. Table 1 lists the ratio of the components of this peptide mixture. As expected, the peptide pairs containing the same amino acids (e.g., Lys(Phe)-Lys(Ala)-NH_2 and Lys(Ala)-Lys(Phe)-NH_2) co-eluted, resulting in higher peak areas than the single peptides. Furthermore, peptides containing phenylalanine have a stronger UV-absorption due to the aromatic ring, also resulting in increased peak areas. Accordingly, the peaks representing the peptide pairs Lys(Phe)-Lys(Ala)-NH_2/Lys(Ala)-Lys(Phe)-NH_2 and Lys (Phe)-Lys(Ile)-NH_2/Lys(Ile)-Lys(Phe)-NH_2 were the strongest, followed by the peak representing the peptide Lys(Phe)-Lys(Phe)-NH_2.

After the X positions had been introduced, the Dde group was removed from the ε-amino group of lysine in position one, and the O position was introduced through the separate acylation of this amino group in each peptide mixture with one of the 20 proteinogenic amino acids or one of the 10 carboxylic acids. The peptide mixtures were cleaved from the resin by HF after deprotection of the side chains of the amino acids in the (X) and (O) positions by TFA.

The synthesis of the second and third positional libraries, *cyclo*[Lys(X)-Lys(O_2)-Lys(X)-Glu]-Gly-OH and *cyclo*[Lys(X)-Lys(X)-Lys(O_3)-Glu]-Gly-OH, was carried out identically with respective modification in the order of Lys(Boc) and Lys(Dde) couplings.

An individual control peptide, *cyclo*[Lys(Ac-Phe)-Lys(Ac-Phe)-Lys(Ac-Phe)-Glu]-Gly-OH, was synthesized along with the library. Besides serving as a control peptide for the course of the synthesis, this peptide was also used to study the enzymatic stability of the library components. The purified peptide was separately incubated with trypsin, chymotrypsin and proteinase K for 15 hours. The RP-HPLC profiles of the peptide before and after incubation with these enzymes were identical, indicating complete stability to hydrolysis by all three enzymes. For trypsin, this result was anticipated due to the modification of the lysine side chains, which would be expected to prevent interaction of the peptide with the active site of trypsin. For chymotrypsin and proteinase K, on the other hand, the complete stability was unexpected, since both enzymes are known to cleave peptide and other amide bonds -P_1 - P_1'- with aromatic amino acids in the P_1 position.

Library Screening

The screening of the cyclic peptide template library, as well as the identifi-

cation of active individual compounds from the library, are illustrated using chymotrypsin as a model target. The 90 mixtures making up this positional scanning library (30 for each of the three positional libraries) were separately tested for their ability to inhibit the chymotryptic hydrolysis of the chromogenic substrate *N*-succinyl-L-phenylalanine-*p*-nitroanilide. As can be seen from the screening data (Figure 6), the same carboxylic acids (i.e., 3 = piperonylic acid, and 9 = 2-thiophenecarboxylic acid) were found to be the most effective functionalities at each of the three positions (lysine side chains). Eight individual compounds representing all possible sequence combinations of these N^{ε}-substituted lysines were synthesized and tested for their chymotrypsin inhibitory activity. Furthermore, the linear analogs of two of these compounds, as well as truncated analogs of the linear sequences, were synthesized and tested in order to evaluate the significance of the cyclic structure for the inhibitory activity, as well as to establish the minimal molecule size necessary for chymotrypsin inhibition. The inhibitory activities of the cyclic and linear compounds are listed in Table 2, along with the most active chymotrypsin inhibitor identified through the screening of an all-D-amino acid hexapeptide library (Ac-ygyyyr-NH_2, unpublished), and chymostatin, a low molecular weight (ca. 550) peptide-aldehyde chymotrypsin inhibitor from microbial origin (49).

The inhibitory activities of the cyclic compounds, although approximately 30-fold lower than that of chymostatin, are comparable to the all D-amino acid chymotrypsin inhibitor. The linear analogs of *cyclo*[Lys(9)-Lys(3)-Lys(9)-Glu]-Gly-OH and *cyclo*[Lys(9)-Lys(3)-Lys(3)-Glu]-Gly-OH (i.e., Ac-Lys(9)-Lys(3)-Lys(9)-Gly-OH and Ac-Lys(9)-Lys(3)-Lys(3)-Gly-OH) are approximately 10 times less active than their cyclic analogs, indicating the importance of the cyclic structure in spite of the relatively long distance between the carboxylic acid residues and the cyclic template (see Figure 1). The inhibitory activity of the linear compounds decreases even more when the sequences are truncated by one or two lysine residues (e.g., Ac-Lys(3)-Lys(9)-Gly-OH and Ac-Lys(9)-Gly-OH).

It should be noted that the cyclic compounds have no structural resemblance to any known natural or synthetic chymotrypsin inhibitors, indicating the potential of SCLs for the identification of novel enzyme inhibitors. Moreover, none of these compounds would have been identified if only the proteinogenic amino acids had been used as building blocks for the library.

This example demonstrates the potential for the rapid identification of individual compounds using positional scanning libraries made up of building blocks other than the proteinogenic amino acids. The non-peptide libraries described thus far are composed of individual compounds bound to different types of solid supports, which are either spatially separated [e.g., plastic pins (3) or resin in tubes (6)], or mixtures of resin beads having one compound per bead (31). The first approach can be considered a multiple synthesis method for individual chemical compounds, analogous to the multiple synthesis methods developed for peptides over the past decade (10,13–15,17,20), rather than a combinatorial library approach. The one bead–one compound method, on the other hand, when applied to the synthesis of libraries composed of building blocks other than the proteinogenic amino acids, requires the presence of peptide (26,34), oligonucleotide (2,33) or other (36) tags that encode the structure of the compound on each bead, which can then be decoded by sequencing or other analysis (e.g., gas chromatography, HPLC) of the coding tag. This, however, requires an additional, independent chemistry for the assembly of the coding tag, so as not to interfere with the synthesis of the library. In contrast, the systematic array (i.e., the use of defined and mixture positions) of SCLs, in particular positional scanning SCLs, eliminates the use of any type of coding as a means of structure determination, since the identification of the structure of the active compounds of interest is inherent to the SCL approach.

CONCLUSIONS

The synthetic combinatorial library concept was utilized for the synthesis of a cyclic peptide template SCL. The positional scanning format permits the identification of the most effective amino or carboxylic acid residues in a single screening, and also eliminates the need for any type of coding for building blocks other than the proteinogenic amino acids. While maintaining peptide character, the components of this library are stable towards proteolytic degradation and represent an increased and unique chemical diversity. Compounds with chymotrypsin inhibitory activity, which bear no structural resemblance to any known chymotrypsin inhibitors, were identified through the screening of this library.

The library described here is the first example of a combinatorial library based on cyclic peptide templates. This concept is being extended to prepare different libraries with respect to the building blocks used for the template (e.g., ornithine, diaminobutyric acid, aspartic acid) as well as for the diversity positions (e.g., D- and other non-proteinogenic amino acids, other carboxylic acids), including the possibility of post-synthetic modification of the peptide backbone, e.g., *N*-alkylation (37) or reduction of the peptide backbone.

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